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学位論文題名

Mannan-degrading enzymes from the common sea hare, *Aplysia kurodai*

（アメフラシ *Aplysia kurodai* のマンナン分解酵素に関する研究）

学位論文内容の要旨

Mannan-degrading enzymes are mainly produced by a wide range of microorganisms from fungi to bacteria, and play a key role in the provision of energy for such organisms. Besides microorganisms, several gastropods are known to produce mannan-degrading enzymes. But these gastropod enzymes have not been so well investigated as bacterial and fungal enzymes. *Aplysia kurodai*, a typical herbivorous marine gastropod, feeds on a variety of red and green seaweeds which contain β-1,4-mannan as a structural polysaccharide. This gastropod is known to possess various polysaccharide-degrading enzymes that digest seaweeds' cell-wall materials providing sugars available as carbon/energy sources. Thus, mannan-degrading enzymes also seem to play important roles for the assimilation of seaweeds' in this animal. Therefore, in the present study, the author investigated the general properties of mannan-degrading enzymes; i.e., endo-β-1,4-mannanase (EC 3.2.1.78; β-mannanase) and β-D-mannosidase (EC 3.2.1.25; β-mannosidase), in the digestive fluid of *A. kurodai*; and determined their primary structures by cDNA cloning.

In chapter 1, purification and characterization of β-mannanase from *A. kurodai* are described. A β-mannanase, named AkMan in the present study, was purified from the digestive fluid of *A. kurodai* by ammonium sulfate fractionation followed by successive column chromatographies on TOYOPEARL phenyl-650M, TOYOPEARL CM-650M and Mono-S 5/50 GL. The purified enzyme was characterized regarding molecular mass, enzymatic properties, substrate specificity and partial amino-acid sequences. AkMan showed a single band of approximately 40 kDa on SDS-PAGE. It showed a broad pH optimum at around 4.0-7.5 and an optimum temperature at 55°C. This enzyme was heat stable and 50% of the original activity was remained after the incubation at 52°C for 20 min. AkMan was also stable in the acidic pH conditions, i.e., the activity practically unchanged at a pH range from 4.0 to 7.5 by the incubation at 40°C for 20 min. Among the metal ions, Fe^{3+} and Ag^{+} caused moderate inhibition on AkMan; however, no considerable inhibition was

caused by the other metal ions tested. Whereas, the activity was appreciably increased in the presence of Co^{2+}, Cu^{2+}, Mn^{+2}, DTT and 2-mercaptoethanol. AkMan most preferably degraded a linear β-1,4-mannan from a green algae *Codium fragile* producing tri- and disaccharides, then glucomannan from konjak root as well as galactomannan, while could not degraded carboxymethyl cellulose, agarose, dextran and xylan. These results indicate that AkMan is a specific enzyme to mannan polysaccharides and regarded as a typical endo-β-1,4-mannanase (EC 3.2.1.78) which splits internal β-1,4-mannosidic linkages of β-mannans. According to the action of AkMan to mannooligosaccharides, the spatial expanse of substrate binding site of this enzyme was considered to be in the size of mannopentaose-mannohexaose unit. The N-terminal and internal amino-acid sequences of AkMan showed high similarity to those of other molluscan enzymes which belong to glycosyl-hydrolase-family 5 (GHF5). Thus, AkMan was also considered to be a member of GHF5.

In chapter 2, cDNA cloning and bacterial expression of AkMan are described. A cDNA encoding AkMan was amplified by RT-PCR from *A. kurodai* hepatopancreas cDNA. The cDNA that includes entire translational region of AkMan consisted of 1,392 bp and encoded an amino-acid sequence of 369 residues. The N-terminal region of 17 residues of the deduced sequence except for the initiation Met was regarded as the signal peptide of AkMan and the mature enzyme region was considered to comprise 351 residues with a calculated molecular mass of 39,961.96 Da. The sequence analysis showed that AkMan belongs to GHF5 like other molluscan β-mannanases. The catalytic residues which act as nucleophiles or proton donors in GHF5 enzymes are conserved in AkMan as Glu162 and Glu293. Phylogenetic analysis for the GHF5 β-mannanases indicated that AkMan together with other molluscan β-mannanases formed an independent clade of the subfamily 10 in the phylogenetic tree. Accordingly, AkMan was confirmed as a new member of GHF5-subfamily 10. The AkMan cDNA was subcloned into pCold I expression plasmid and expressed in *Escherichia coli* BL21(DE3) as a recombinant fusion protein possessing a hexahistidine-tag at the N-terminus. The recombinant AkMan purified by Ni-NTA affinity chromatography showed a broad pH optimum similar to native AkMan; however, temperature optimum and thermal stability was considerably lower than those of native enzyme. The actual reason for such differences between the recombinant and native enzymes is currently obscure; however, it may be possible to consider that the folding structure of recombinant enzyme is somewhat difference from that of native enzyme. The substrate preference of AkMan and the degradation products of β-mannan and mannooligosaccharides produced by recombinant enzyme

were comparable to those of native enzyme.

In chapter 3, purification and characterization of a β-D-mannosidase from *A. kurodai* was described. The β-mannosidase, AkMnsd, was purified from the digestive fluid of *A. kurodai* by ammonium sulfate fractionation, TOYOPEARL Butyl-650 M, TOYOPEARL DEAE-650 M, and Superdex 200 10/300 GL chromatographies. The enzyme has a molecular mass of approximately 100 kDa. The enzyme showed maximum activity on *p*-nitrophenyl β-D mannopyranoside at pH 4.5 and 40°C and found to be stable in the acidic pH range from 2.0 to 6.7 and the temperature below 38°C. The *K*m and *V*max values for AkMnsd determined at pH 6.0 and 30°C with a substrate *p*-nitrophenyl β-D-mannopyranoside were 0.10 mM and 3.75 μmoL/min/mg, respectively. The β-mannosidase hydrolyzed β-1,4-linked mannan and mannooligosaccharides and released mannose residues from the substrates. The hydrolysis pattern clearly indicated that AkMnsd acted on these substrates in an exolytic manner. In the presence of the *Aplysia* β-mannanase AkMan, the production of mannose from linear β-1,4-mannan by AkMnsd was considerably improved compared with the production by AkMnsd alone. These results suggested that both two enzymes, i.e., β-mannanase and β-mannosidase, are necessary to hydrolyse β-1,4 mannan efficiently.

In chapter 4, cDNA cloning of AkMnsd is described. A cDNA encoding AkMnsd was amplified from the *Aplysia* hepatopancreas cDNA by the PCR using degenerated primers designed on the basis of N-terminal and internal amino-acid sequences of AkMnsd. The cDNA consisted of 2,985 bp and encoded the sequence of 931 amino-acid residues. The molecular mass of mature AkMnsd calculated from the deduced sequence was 101.97 kDa. The sequence of AkMnsd shared 20-43% amino-acid identity to those of GHF2 β-mannosidases. The catalytically important amino-acid residues determined in GHF2 enzymes were completely conserved in AkMnsd. Thus, AkMnsd is regarded as a new member of GHF2 mannosidase from marine gastropod. As far as authors' knowledge, this is the first report on the cDNA cloning for invertebrate β-mannosidase.

In conclusion, this study revealed that endo- and exo-acting mannan-degrading enzymes, AkMan and AkMnsd were present in the digestive fluid of *A. kurodai*. By the synergistic action of these enzymes, *A. kurodai* can depolymerize the ingested seaweed mannan efficiently to D-mannose which can be assimilated by this animal as a carbon and energy source.

学位論文審査の要旨

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学位論文題名

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（アメフラシ *Aplysia kurodai* のマンナン分解酵素に関する研究）

海洋無脊椎動物の食物消化機構の解明とそれに関連する酵素のバイオテクノロジーへの活用に関心が高まっている。マンナン分解酵素はエンド型とエキソ型のものに大別され，マンナンを含有する各種の飲料や食品，餌料などの品質改善に有用であることから，産業的にも広く利用されている。マンナンは，マンノースの重合した多糖であり，その内部領域のグリコシド結合を加水分解するものが，エンド型マンナナーゼ（EC 3.2.1.78）と定義される。一方，マンナンやマンノオリゴ糖の非還元末端に作用してマンノースを遊離するものが，マンノシダーゼ（エキソ型マンナナーゼ、EC 3.2.1.25）である。

本論文は，食植性腹足類の一種であるアメフラシ *Aplysia kurodai* の消化液に含まれるマンナナーゼおよびマンノシダーゼを，疎水クロマトグラフィーやイオン交換クロマトグラフィーをはじめとする種々の分離方法を駆使することによって単離し，それらの生化学的性状の解析，cDNA クローニングによる一次構造の解析およびマンナナーゼに関しては大腸菌を用いた組換え酵素の発現生産を行ったものである。この研究成果により，腹足類のマンナン分解酵素に関する生化学的および分子生物学的な知見を世界で初めて得ることができた。さらに，アメフラシの摂餌した海藻（特に緑藻と紅藻）に含まれるマンナンが両酵素によって効率的にマンノースにまで分解されることを証明するとともに，この生じた単糖がこの海洋動物の炭素・エネルギー源として利用されている可能性を示した。本研究成果の概要は以下の通りである。

1. アメフラシの消化液から分子量 40,000 のエンド-β-1,4-マンナナーゼ AkMan を単離することに成功した。AkMan は至適温度が 55°C で pH 4.0-7.0 の広い pH 域で高い活性を示すという既報の軟体動物のマンナナーゼには見られない特徴を有していた。また，AkMan は緑藻ミル由来の直鎖マンナンだけでなく，陸上植物由来のグルコマンナンやガラクトマンナンも良く分解した。さらに，種々のオリゴ糖基質に対する分解活性を比較することにより，AkMan の活性部位の大きさは 5 糖～6 糖に相当することを明らかにした。
2. アメフラシの肝膵臓の mRNA から合成した cDNA から，PCR により AkMan をコードする cDNA をクローン化することに成功した。この cDNA は 1392 bp から成り，その翻訳領域から 369 残基の

アミノ酸配列が翻訳され，理論分子量は39961.96と計算された。AkManのアミノ酸配列を，細菌，真菌，植物および無脊椎動物のマンナナーゼのアミノ酸配列と比較することにより，本酵素がglycosyl-hydrolase-family 5 (GHF5)のsubfamily 10に属することを明らかにした。

3. 大腸菌の低温誘導発現系を利用することにより，組換えAkManの生産に成功した。組換え酵素は天然AkManと同様，pH 4.0-7.0で高い活性を示し，直鎖マンナンやガラクトマンナンを分解可能であるが，温度安定性は低下することを明らかにした。
4. アメフラシの消化液から，β-マンノシダーゼAkMnsdを単離することに成功した。本酵素は，マンノオリゴ糖やpNP-マンノシドを良く分解し，AkMan共存下で直鎖マンナンやガラクトマンナンを効率的に分解しD-マンノースを生じた。このことから，アメフラシはマンナナーゼとマンノシダーゼを用いて海藻のマンナンをマンノースに分解し，炭素源として利用していると推定した。
5. AkMnsdのcDNAをクローン化することに成功した。このcDNAは2985 bpから成り，931残基のアミノ酸配列をコードしていた。このcDNAは，軟体動物で初めてクローン化されたマンノシダーゼの遺伝子であった。このcDNAから演繹されるアミノ酸配列から，AkMnsdはGHF2に属する酵素であることを明らかにした。

以上，本研究は海産腹足類アメフラシのマンナン分解酵素としてβ-1,4-マンナナーゼとβ-マンノシダーゼの酵素特性と一次構造を明らかにするとともに，β-1,4-マンナナーゼについては大腸菌による組換え酵素の発現系を構築し，本酵素のタンパク質工学的基盤を整備したものとして高く評価できる。よって審査員一同は申請者が博士（水産科学）の学位を授与される資格のあるものと判定した。